Blood donor white blood cell reduction filters as a source of human peripheral blood–derived endothelial progenitor cells

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BACKGROUND: Neovascularization in tumors, wounds, and sites of ischemic injury occurs by both angiogenesis (proliferation from existing vessels) and by vasculogenesis (differentiation into endothelial cells from circulating endothelial progenitor cells [EPCs]). EPCs can be obtained from marrow, from cord blood, or by ex vivo expansion of human peripheral blood (PB). The ease of obtaining human PB EPCs has led many recent studies to utilize PB EPCs. The ability to obtain large numbers of PB EPCs would greatly facilitate characterization to further our understanding of EPC biology and their application in cellular gene therapy.

STUDY DESIGN AND METHODS: Peripheral blood mononuclear cells (PBMNCs) from whole blood or from the material obtained from white blood cell (WBC) reduction filters were isolated. The cells from both sources were then cultured separately under defined conditions to quantify EPC yield from each source.

RESULTS: The yield of EPCs per million PBMNCs plated was approximately 3.5-fold higher from fresh PB. Because greater numbers of PBMNCs were obtained from each filter, however, the mean yield of EPCs from one filter versus fresh blood was 5.4 million versus 0.4 million, respectively (approx. 14-fold increased yield).

CONCLUSION: The use of WBC reduction filters provides a safe, inexpensive, and readily available source for large numbers of PBMNCs from which culture-expanded EPCs can be generated for further study.

Angiogenesis, the formation of new blood vessels from existing vessels, was previously believed to be the sole mechanism of new blood vessel formation during early postnatal development as well as during adulthood.[1] There is now growing evidence, however, that vasculogenesis, the formation of vessels from circulating endothelial progenitor cells (EPCs), also contributes significantly to neovasculature during early postnatal development,[2] ischemic injury,[3] wound repair,[4] and malignant growth.[5,6] EPCs have been shown to preferentially "home" to foci of ischemic or tumor neovascularization, which are associated with high levels of local vascular endothelial growth factor production (VEGF).[2,4,7]

The number of circulating EPCs in humans has been associated with various diseases such that they are considerably lower in certain pathologic conditions, such as diabetes or renal failure.[8-10] Reduction in EPC numbers is believed to contribute to endothelial dysfunction observed in these patient populations.[9] A robust supply of EPCs could, theoretically, ameliorate these deficiencies in neovascularization.[11] Alternatively, high circulating levels of EPCs may enhance tumor growth and, hence, may be undesirable in such situations. Several investigators have

ABBREVIATIONS: DiI-acLDL = 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine-labeled acetylated low-density lipoprotein; EPC(s) = endothelial progenitor cell(s); PB = peripheral blood; UEA-1 lectin = *Ulex europaeus* agglutinin 1; VEGFR2 = vascular endothelial growth factor receptor-2.

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shown that tumors selectively utilize EPCs.[12] Thus, there is growing interest in developing genetically modified EPCs to carry an antiangiogenic therapy as a strategy to slow tumor vasculature.

In vitro, EPCs can be derived from embryonic stem cells,[13] marrow,[14] cord blood,[7] and cytokine-mobilized peripheral blood (PB).[4] More recently, Kalka and colleagues[15] demonstrated that EPCs can be derived from ex vivo expansion of unmobilized human PB.[15] Administration of culture-expanded PB EPCs to mice with hindlimb ischemia resulted in enhanced neovascularization and reduced limb loss.[15] The relative ease of obtaining unmobilized human PB has made PB EPCs an attractive candidate with which to develop cell-based human therapy. Clinical trials such as the TOP-CARE study are designed to investigate the therapeutic role of autologous culture-expanded EPCs after ischemic injury.[16] In parallel with such studies, there is continued effort to better understand EPC biology. The in vivo proliferative potential and homing characteristics of PB EPCs are not yet known. In fact, a growing number of investigators, including our lab, are involved in further study and characterization of the biology of PB EPCs. Although almost all laboratories have access to human PB, research on human subjects requires full review by the institutional review board and necessitates obtaining informed consent from donors. Furthermore, only one to two tubes of blood are usually drawn from normal volunteers, who are often recruited among coworkers and students in the laboratory setting. In general, such volunteers are not routinely tested for many blood-borne pathogens. We describe the isolation of peripheral blood mononuclear cells (PBMNCs) from white blood cell (WBC) reduction filters obtained from the American Red Cross blood donor center. Culture of filter-derived PBMNCs under defined conditions generated EPCs with characteristics comparable to those generated from expansion of PBMNCs isolated from fresh blood.

MATERIALS AND METHODS

Isolation of PBMNCs

Human blood donor WBC reduction filters (LeukotrapRC, Pall Corporation, East Hills, NY) were obtained from the American Red Cross (Nashville, TN) and were kept at 4°C overnight. With a 30-mL syringe, the filters were backflushed once with phosphate-buffered saline (PBS), collecting approximately 25 mL of blood-cell suspension from each filter in a 50-mL conical tube. Resultant blood was diluted 1:1 with PBS. Samples of fresh blood from human volunteers were collected into 8.5-mL acid-citrate-dextrose solution A tubes. Each tube was diluted 1:1 with PBS into a 50-mL conical tube. Total PBMNCs were isolated from both filter and fresh blood by density gradient centrifugation at 740 ×g for 30 minutes at 4°C by overlaying approximately 12 mL of Histopaque 1077 (Sigma Chemical Co., St Louis, MO) with 38 mL of PBS and blood-cell suspension. The PBMNCs were collected from the interface, and viability was determined with trypan blue dye exclusion. A total of 20 filters and four separate fresh blood samples were analyzed for this study. The data shown are representative of multiple experiments, (i.e., fluorescence-activated cell sorting [FACS], immunostaining) or are the means derived from multiple experiments, (i.e., EPC yield).

Cell culture

PBMNCs, isolated as described, were plated at 1×10^6 cells per cm^2 on 100-mm culture dishes or on 22-mm^2 glass coverslips (placed in six-well plates) coated with human fibronectin (Sigma Chemical Co.) diluted 1:50 in Hanks' balance salt solution. These cells were maintained in EC basal medium-2 (Clonetics, San Diego, CA) supplemented with EGM-2 MV SingleQuots. After the third day in culture, nonadherent cells were removed via PBS washings, and fresh EC basal medium-2 was added. Both filter and fresh blood cultures were maintained through Day 14.

Cellular staining

Fluorescent staining of adherent cells on glass coverslips was conducted after 10 days in culture. First, the adherent cells were incubated with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine (DiI)-labeled acetylated low density-lipoprotein (acLDL) (Biomedical Technologies, Stoughton, MA) diluted 1:20 in EC basal medium-2 for 2 hours at 37°C. After fixing these cells with cold acetone, fluorescein isothiocyanate (FITC)-labeled *Ulex europaeus* agglutinin 1 (UEA-1 lectin) (Vector Laboratories, Burlingame, CA) diluted 1:20 in PBS was then applied to the same coverslip for 1 hour. For immunostaining, coverslips were incubated with anti-vascular endothelial growth factor receptor-2 (VEGFR2, also known as flk-1 or KDR) (1:100, Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. The coverslips were washed and incubated with fluorescent-conjugated appropriate fluorescent secondary antibodies for 2 hours. After the cells were washed with PBS, each coverslip was applied to a slide face down with one drop of Vectastain with DAPI (Vector Laboratories). The slides were viewed under an upright fluorescence microscope (Zeiss). For EPC enumeration, the cells demonstrating positive fluorescent costaining of DiI-acLDL and UEA-1 lectin as well as positive fluorescent staining anti-VEGFR2 were identified as differentiated EPCs.

FACS

FACS analysis of PBMNCs was performed by staining 1×10^6 cells in 100 µL with antibodies against CD2, CD14, CD15, or CD19, all conjugated to FITC (Becton Dickinson,

San Jose, CA). For quantitating progenitor cells, cells were coincubated with pooled CD34 antibody (Becton Dickinson) conjugated to phycoerythrin and with antibodies constituting a lineage (lin) cocktail (CD2, CD14, CD15, and CD19). Isotype-identical antibodies served as controls (Becton Dickinson). Nonviable cells identified by 7-aminoactinomycin D (Molecular Probes, Eugene, OR) staining were excluded. Quantitative FACS was performed on a flow cytometer (FACStarPlus, Becton Dickinson) and subsequently analyzed with computer software (Cellquest, Becton Dickinson). At least 10,000 cells were analyzed to obtain histograms of cell number versus logarithmic fluorescence intensity.

Quantitation of EPCs

EPCs were counted with an inverted microscope (Zeiss) in a 10× bright field, of which the plate surface area shown in the eyepiece is 0.985 mm^2. Ten random fields were chosen for each sample, and the number of EPCs was recorded. The number of adherent EPCs per square millimeter on a 100-mm dish (surface area, 78.5 cm^2; Corning, Acton, MA) or adherent to the bottom of a well in a six-well plate (surface area, 9.5 cm^2; Corning) was then multiplied by the respective surface area to achieve the total average number of EPCs present on Day 10 versus the number of PBMNCs initially cultured.

RESULTS

WBC reduction filters were obtained from the American Red Cross Donor Center. Approximately 425 to 500 mL of blood collected containing the anticoagulants AS-3 and CP2D were processed for leukoreduction through the filters. Filters were obtained within 2 hours of collection and MNCs were harvested the next morning. PBMNCs obtained from both fresh PB and WBC reduction filters after density gradient centrifugation were analyzed by FACS. Analyses of forward and side scatter parameters (Fig. 1) are shown from filter-derived cells isolated by the same technique as from fresh human blood. The products differed by two aspects. First, the filter-derived cells consistently had a lower percentage of viable cells compared to fresh product, 78 percent versus 90 percent, respectively, and this difference was significant ($p < 0.01$). Second, the fresh product contained cells of larger size. To evaluate this further, we determined the cellular composition from each source as shown in Table 1. EPCs are considered to originate from CD34+ PB progenitor cells that lack lineage markers (CD34+/lin–).[4,5] The percentage of CD34+/lin– cells derived from each source was similar (Table 1). Similarly, the relative lymphocyte content in the two products was not different. The relative numbers of CD14+ monocytes and CD11b+ granulocytes, however, were significantly higher in the PBMNC product obtained from fresh blood compared to the filters (Table 1). Culture of PBMNCs derived from both sources under conditions that favor EPC growth and adherence resulted in the appearance of elongated cells with endothelial morphology by Day 3. The cells were analyzed on Days 7 and 10 (shown) for the ability to uptake DiI-acLDL and staining with UEA-1 lectin, consistent with endothelial lineage (Fig. 2). Uptake of fluorescently labeled acetylated LDL is a property shared by both macrophages and endothelial cells.[17] By contrast, staining with UEA-1 lectin is consid-

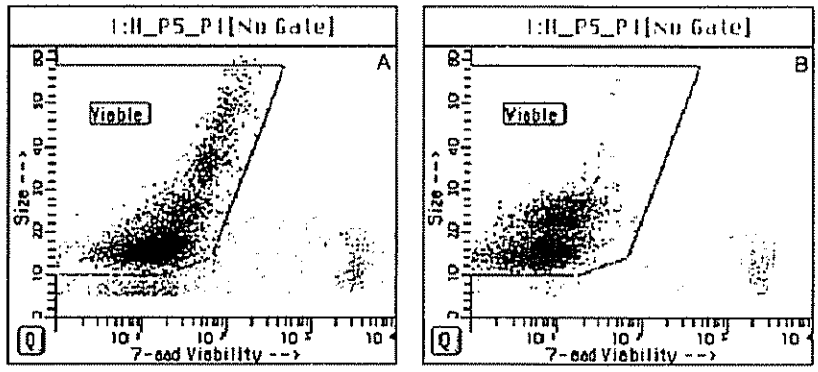

Fig. 1. Flow cytometric analysis of PBMNCs from fresh human blood (A) or from a WBC reduction filter (B). Cells were gated by forward scatter and assessed for viability with 7-aminoactinomycin D exclusion. Further FACS analysis with lineage-specific antibodies were performed on gated viable cells. These data are summarized in Table 1.

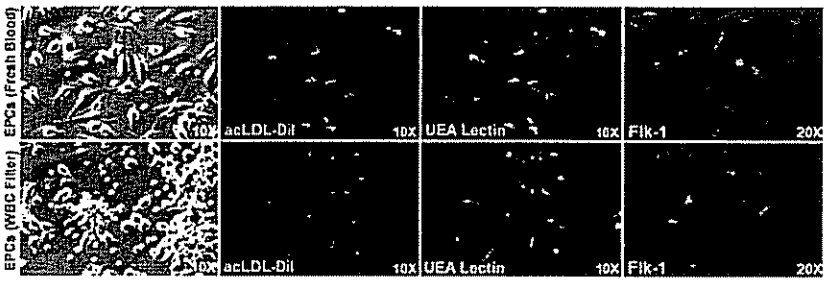

Fig. 2. In vitro differentiation of PBMNCs into EPCs. Bright-field images of EPCs generated from PBMNCs after 7 days in culture are shown in left top and bottom panels. The two middle panels demonstrate the ability of culture-expanded EPCs to endocytose DiI-acLDL (red fluorescence) and to bind UEA-1 lectin (green fluorescence), respectively. The last panel shows immunofluorescent staining with VEGFR2 (Flk-1) antibody.

TABLE 1. Cellular composition from each source*

Source	% Viable	CD2 (T cells)	CD14 (monocytes)	CD15 (granulocytes)	CD19 (B cells)	CD34+/lin– (progenitor cells)
WBC filter	78 ± 5†	66 1 ± 13	12 5 ± 3†	3 7 ± 2†	17 8 ± 5	0 05 ± 0 2
Fresh blood	90.2 ± 4	73.5 ± 2	34.1 ± 7	11.7 ± 3	14.6 ± 8	0.04 ± 0.02

* Data obtained from FACS analysis of MNCs isolated from the sources as designated
† Significance with the t test

ered specific for endothelial lineages.[18] These have been utilized to identify EPCs in previous publications.[9,19-22] To further confirm endothelial lineage, the expanded cells were also stained with marker VEGFR2 (also known as flk-1 or KDR; Fig. 2).[23] To determine the utility of use of filters as a source for EPCs, we compared the numbers of EPCs obtained by Day 10 after culture from each source. The numbers of PBMNCs isolated from one filter was considerably higher (approx. 33-fold) than what was isolated from one tube (approx. 7.5 mL) of fresh blood (Fig. 3A). The efficiency of EPC growth, however, was significantly higher from fresh PB than filters by approximately 3.5-fold ($p < 0.02$; Fig. 3B). With these data, we calculated the total EPC yield from one filter (5.4 million) versus one tube of fresh blood (0 4 million) (Fig. 3C), a difference of approximately 14-fold.

DISCUSSION

We demonstrate that culture expansion of PBMNCs obtained from WBC reduction filters under appropriate conditions resulted in the growth of EPCs. This supports the use of WBC reduction filters, in lieu of fresh human blood, as a source of culture-expanded EPCs. The outgrowth of EPCs was evident as early as Day 3 from both sources with large clusters of EPCs evident between Day 7 and Day 14. Although the CD34+/lin– content of PBMNCs obtained from fresh blood was nearly identical to those obtained from filters, the efficiency of EPC generation was approximately 3.5-fold higher from fresh product. This discrepancy may, in part, be related to the higher viability (approx. 13% greater) of the fresh blood source. Also, the monocyte and granulocyte content of the PBMNCs obtained from fresh blood was significantly higher (approx. 3-fold). It has been previously reported that culture of a highly purified population of CD34+/lin– cells compared to unfractionated marrow resulted in greater than 10-fold lower EPC yield.[4] This suggests that accessory cells and/or cytokines may be present in the nonprogenitor population that facilitates EPC expansion and differentiation in culture. Monocytes, for example, may serve as such accessory cells, and their increased numbers may improve EPC expansion.[24] Despite the increased efficiency observed with fresh blood cells, the total numbers of EPCs obtained from one filter is far greater (approx. 14-fold) than that obtained from one tube of fresh human blood

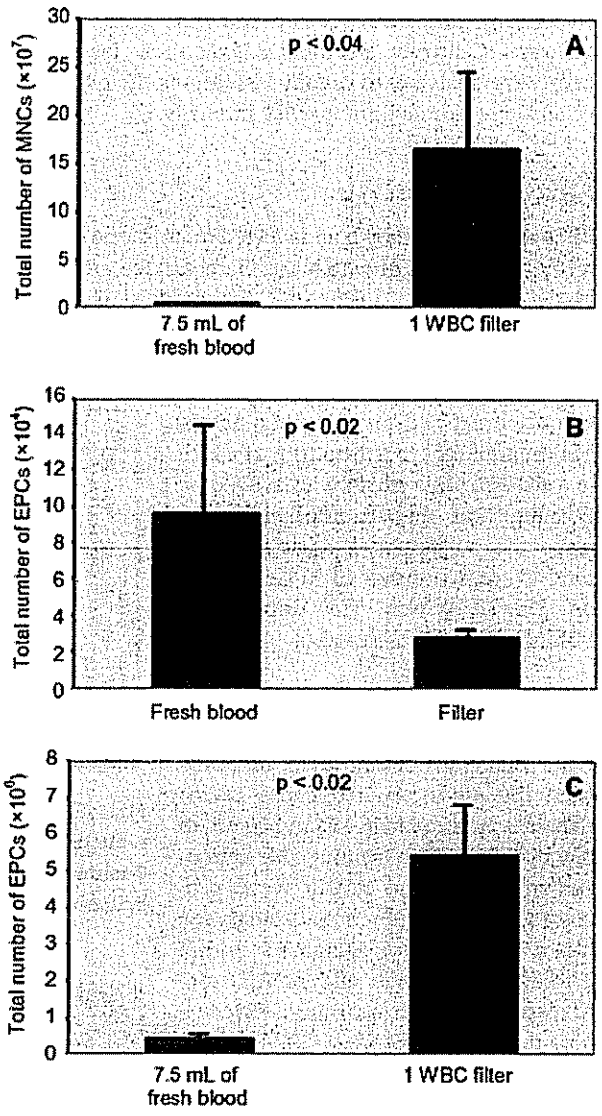

Fig. 3. EPC yield from WBC filter versus fresh PB. (A) MNC yield; (B) EPC yield per 10^6 MNCs plated; (C) total EPC yield per source. p values were obtained with the t test and $p < 0.05$ is considered significant in our study.

(approx. 7.5 mL). Transplantation of human cells or tissues into immunodeficient mouse models provides a tool by which the biology of human cells can be studied with animal models.[4,25] Our laboratory is utilizing a NOD/SCID

mouse in combination with heterotopic tumor growth to study the biology of human EPCs during malignant neovascularization (Young and Teleron, unpublished studies). The use of EPCs for in vivo studies with animal models of ischemia or tumor growth necessitates approximately 0.5×10^6 to 1×10^6 EPCs administered per mouse.[4] The ability to generate large numbers of EPCs will significantly facilitate these studies.

As always, the use of any human product, including filters, necessitates conscientious adherence to universal precautions. Obtaining filters from a blood donor center, however, adds an added measure of safety because the volunteer donors are questioned about risk factors for blood-borne pathogens before donation. Each filter carries no identifying donor information and thus is unlinked to a specific donor. Hence, the use of discarded filters is not considered human subjects research, does not require patient consent, and is considered exempt from full approval and review by the institutional review board of the American Red Cross and Vanderbilt University Medical Center. Were these filters not used in this study, they would have been destroyed as biohazard waste.

In summary, the use of blood donor WBC reduction filters with a simple back-flush manipulation provides a safe, inexpensive, and readily available source for large numbers of human PBMNCs. These can subsequently be expanded in culture to generate EPCs for further study.

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